Molecular Cloning of Somatotropin Gene in Empurau (*Tor tambroides*)

Dorathy Anak Jampi
(34804)

Bachelor of Science with Honours
(Resource Biotechnology)
2015

Acknowledgement

This project was performed in partial fulfilment of Bachelor's Degree of Science with Honours. Foremost, I would like to express my sincere gratitude to my supervisor, Dr Lee Kui Soon for the continuous support, patience and motivation throughout this project. It was a valuable opportunity to be able to work on this project under his guidance.

Besides, I would like to thank my family, especially my father and mother, for their continuous support and moral advice throughout this project even though the miles apart us. Thank you also for giving me the financial support and encouragement that I needed.

My sincere thanks also goes to all the postgraduate students that helped me during this project. I learned a lot from them and gained more experience in conducting laboratory works. Last but not least, I want to thank my fellow laboratory mates and friends for always being together through hardship and joyful moments in the laboratory.

Declaration

I, Dorathy anak Jampi, 34804, Faculty of Resource Science and Technology, hereby declare that the work entitled Molecular Cloning of Somatotropin Gene in Empurau (*Tor tambroides*) is my original work. I have not copied from any other students' work or from any other sources except where due reference or acknowledgement is made explicitly in the text, nor has any part been written for me by another person.

30 JUNE 2015

Date submitted

Dorathy anak Jampi (34804)

Table of Contents

List of Abbreviations

A_{280}	Absorbance at 280 nm
A_{260}	Absorbance at 260 nm
A_{230}	Absorbance at 230 nm
AGE	Agarose Gel Electrophoresis
BLAST	Basic Local Alignment Search Tool
bp	base pairs
IGF-1	Insulin-like Growth Factor-1
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IUCN	International Union for Conservation of Nature
LBAIX	Luria Broth/Ampicillin/IPTG/X-Gal
RT-PCR	Reverse Transcription Polymerase Chain Reaction
ST	Somatotropin
UV	Ultraviolet
V	Volts
X-gal	5-bromo-4-chloro-3-indolyl β-galactopyranoside

List of Tables

List of Figures

Molecular Cloning of Somatotropin Gene in Empurau (*Tor tambroides*)

Dorathy anak Jampi

Resource Biotechnology
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

ABSTRACT

Somatotropin, also known as growth hormone, is a peptide hormone that stimulates cell reproduction, regeneration and growth in organisms. In fish, somatotropin regulates the growth and differentiation of skeletal muscle. *Tor tambroides* is known to be used for delicacy, ornamental and economic activities. The growth rate of *T. tambroides* is about 500 g per year, followed by 1 kg to 2 kg per year after reaching weight between 2 kg to 3 kg and could reach up to 50 kg to 60 kg. Due to its slow growth rate, the price of the fish is relatively high. However, there is lack of information on *T. tambroides* somatotropin gene. Thus, this study is important in order to make numerous clones of the gene that could be molecularly applied for retrieving more information and providing an insight on gene alteration for enhancing its growth performance in aquaculture practices. *T. tambroides* somatotropin gene was successfully isolated and sequenced. The gene was obtained through total RNA isolation and converted to cDNA using RT-PCR. Primer was designed to amplify the DNA fragment and cloned into pGEM®-T Easy Vector. The glycerol stock of *Escherichia coli* containing the plasmid was stored for further uses such as probe preparation for expression study.

Keywords: *Tor tambroides, somatotropin,* sequence, RT-PCR, growth, primer

ABSTRAK

Somatotropin, juga dikenali sebagai hormon pertumbuhan, adalah hormone peptide yang merangsang pembiakan sel, pertumbuhan semula dan pertumbuhan dalam organisma. Dalam ikan, somatotropin mengawal pertumbuhan dan pembezaan otot rangka. Tor tambroides terkenal dengan penggunaannya dalam sajian istimewa, hiasan dan aktiviti ekonomi. Kadar pertumbuhan T. tambroides adalah kira-kira 500 g setiap tahun, diikuti dengan 1 kg hingga 2 kg setiap tahun selepas mencapai berat badan antara 2 kg hingga 3 kg dan boleh mencapai sehingga 50 kg ke 60 kg. Disebabkan oleh kadar pertumbuhan yang perlahan, harga ikan tersebut agak tinggi. Walau bagaimanapun, terdapat kekurangan maklumat mengenai gen somatotropin T. tambroides. Oleh itu, kajian ini adalah penting untuk membuat banyak klon gen yang boleh diaplikasikan secara molekul untuk mendapatkan maklumat lanjut dan menyediakan gambaran pada gubahan gen untuk meningkatkan prestasi pertumbuhan ikan tersebut dalam amalan akuakultur. Gen somatotropin T. tambroides telah berjaya diasingkan dan dijujuk. Gen itu telah diperoleh melalui pengasingan RNA total dan diubah kepada cDNA menggunakan RT-PCR. Primer direka untuk memperbanyakkan cebisan DNA dan diklon ke pGEM®-T Easy Vector. Stok gliserol Escherichia coli yang mengandungi plasmid telah disimpan untuk digunakan lagi seperti dalam penyediaan probe untuk kajian ekspresi.

Kata kunci: Tor tambroides, somatotropin, jujukan, RT-PCR, pertumbuhan, primer

1. Introduction

The King of Sarawak rivers, *Tor tambroides,* also known as Empurau, Mahseer and Wang Bu Liao is a fish belongs to Cyprinidae family. The fish inhabits clean rivers and lakes with moving streams and breed at rocky river bottom. Kapit division is a famous place to obtain this fish because of its suitable geographical features which is located at the upper stream of Rajang River.

T. tambroides can be identified by its large scales and unique lower lips that have long median lobe connected to its corners of mouth (Haryono & Tjakrawidjaja, 2005). The eggs colour is pale yellow, medium or dark golden orange whereas the adult fish can be seen in reddish or silver-bronze colour (Haryono & Tjakrawidjaja, 2005). *T. tambroides* is an omnivorous fish that feed on smaller fish, insects and illipe nuts (engkabang fruit).

There is scarce information about *T. tambroides* growth rate based on somatotropin (ST) gene. The growth rate in the first three years is about 500 g per year, follow by 1 kg to 2 kg per year after reaching weight between 2 kg to 3 kg and can reach about 50 kg to 60 kg in lifetime (Wong, 2010). Besides, it is also listed in IUCN Red List Status of Data Deficient (DD) due to human activities such as overfishing for commercializes consumption, logging and poaching (Kottelat, 2012).

As a high demand fish in Malaysia and other countries like China and Hong Kong, the slow growth rate of *T. tambroides* will definitely becoming a problem for commercial breeding process. The taste of the flesh which is sweet, edible scale that highly contained with collagen and no muddy smell due to its diet of illipe nuts are the main factors for its famousness (Ong, 2012). *T. tambroides* is also harmless to human.

Hence, this study is important for future aquaculture industry of high priced *T. tambroides* in which can reach about RM600 per kg for weight of 1.0 kg - 1.5 kg and RM750 per kg for weight of more than 2.0 kg according to LTT Aquaculture Sdn Bhd managing director, Bill Lu Thian Tack on his interview with StarBiz (Wong, 2010). Hence, the alteration of ST gene can increase the rate of growth in *T. tambroides* and produce mature or adult fish in shorter time. This will provide more affordable price, bigger number of production for export purposes and contribute to the economy of the country.

Proportional to the study, further knowledge about the somatotropin gene that controls the growth pattern of other vertebrates, especially in *T. tambroides* should be conducted as the gene data is limited. Therefore, this study is aim to:

a) To clone the somatotropin gene of *Tor tambroides*
b) To sequence and identify the somatotropin gene of *Tor tambroides*

1.1. Problem Statement

The slow growth rate of *Tor tambroides* is the main concern in this study. This study is conducted because there is scarce information of somatotropin (ST) gene in *Tor tambroides* that becomes limiting factor for growth rate enhancement research to be conducted in this species. The growth rate can be enhanced by directly altering the gene regulation or through injection of ST into the fish. Besides, *T. tambroides* is also showing a decreasing number in unknown pattern and listed in IUCN Red List Status of Data Deficient. Thus, this study will retrieve more data and show the expression pattern of ST gene in *T. tambroides*.

2. Literature Review

2.1. History of Somatotropin Gene

The pituitary gland first being known for its significance for growth process was in late nineteenth century (Ayyar, 2011). Unfortunately, the use of growth hormone (GH) which was isolated from the pituitary gland was limited back then as it was hard to isolate the hormone. Nowadays, the improvement in science and technology has developed an easier way to get the source of particular GH via recombinant biotechnology.

Impurities and mixture of other hormones have become a problem to isolation process of GH from the pituitary gland. After a few failures, finally the first pure GH was isolated from pituitary gland of an ox in 1994 by Choh Hao Li and Evans (Sneader, 2005). 'Somatotropin' (ST) is a later term which has been used to depict GH (Sneader, 2005). ST is species specific in nature as a study in 1954 by Grace Pickford showed that inactivation of the fish ST in rat but activation in fish itself (Sneader, 2005). The different physicochemical properties of ST is responsible for this action but in some cases such as rat that response to human, bovine, simian, ovine, and cetaceous ST showed that there are similarities although isolated from different species (Matsuzaki & Raben, 1965).

2.2. Significance of Somatotropin Gene

Somatotropin (ST) regulates the growth and differentiation of skeletal muscle in fish. It can be found abundantly in the liver, skeletal muscle and peripheral tissue (Clemmons, 2014). ST gene is mainly concern about growth process that involved the stimulation of IGF-1 that induce proliferation of chondrocytes for bone growth, myoblasts for muscle growth and stimulate the amino acid uptake and protein synthesis. Besides, ST also acts on metabolic process of protein, fat and carbohydrate.

The study of ST in animals and human is widely ongoing now because of its significance in medical field and economic. Starting from year 1985, the recombinant ST was manufactured by many companies in pharmaceutical industry. In fish especially, the function of this study can clearly see as significant that application of synthetic ST in animals (carp, catfish and tilapia) showed 60% to 600% enhancement in size and growth rate in Thomas Chen and colleagues study of rainbow trout ST gene (Sherman, 2002). In addition, the growth enhancement of transgenic tilapia that contained exogenous piscine ST gene also increase significantly about 3 times average weight in nontransgenic even though in G1 and G2 generation (Rahman & Maclean, 1999). Directly, the cost and time needed for commercial aquaculture industry to wait for fish to reach market size will be reduced significantly.

As widely known, *Tor tambroides* is one of the high demand fish in Malaysia aquaculture industry due to its uniqueness in taste and market-valued. Unfortunately, the limiting factor in study of ST gene in *T. tambroides* is the scarce of ST gene information and data. The alteration of ST gene to enhance its growth rate will reduce its cost in feeding and retail-price. Furthermore, it also helps in curing the fact that *T. tambroides* is reducing in numbers nowadays mostly due to human activities.

2.3. Previous Studies of Somatotropin

Advancement and improvement in science and technology are beneficial in aquaculture industries. Biotechnology knowledge helps scientist to understand the biological processes in animal body and enhance its processes. Based on economic value, growth rate plays a major role in determining the aim of a study.

In the last few years, *Tor tambroides* has been studied to increase its growth rate. Some of the study conducted is based on the feeding system to determine the optimum diet required by *T. tambroides.* A study funded by Ministry of Science, Technology and Innovation (MOSTI) Malaysia had been conducted to determine the optimum level of protein diet in order to compensate the arginine resource deficiency for metabolic processes of *T. tambroides* (Misieng et al., 2011). On the other hand, instead of changing the diet of *T. tambroides,* direct manipulation of the somatotropin (ST) gene that coding for the growth hormone (GH) should be conducted to gain more direct and completely positive effect on its growth rate. The study of ST gene of *T. tambroides* have not yet developed but it can be done based on few studies of the gene in other species.

2.3.1. Somatotropin Analysis in *Piaractus mesopotamicus*

The study of somatotropin (ST) in freshwater fish, *Piaractus mesopotamicus* that live in neotropic ecozone is a good example for this study. RT-PCR is used to amplify the ST cDNA that isolated from pituitary cells mRNA. The result showed sequences that contained 10 amino acids gaps at the N-terminus of the assumed ST polypeptide and the amplified cDNA showed 543 nucleotides that encoded 178 amino acids of the ST (Pinheiro et al., 2008). The sequences also can be used to determine its taxonomy.

2.3.2. Somatotropin Analysis in Indian Major Carps

Different method is conducted through the study of somatotropin (ST) cDNA in Indian major carps. The isolated ST cDNA is amplified using the modified rapid amplification of cDNA ends (RACE) and expressed in *Escherichia coli.* The result showed 1146 nucleotides that encoded 210 amino acids of the ST in *Cirrhina mrigala* and 1156 nucleotides that encoded 211 amino acids of the ST in *Catla catla* (Venugopal et al., 2002). Besides, the proteins

secondary structure is analysed using the genomics (generic and dynamic) expression to predict its algorithms.

2.3.3. Somatotropin Analysis in *Heteropneustes fossilis*

A study of somatotropin (ST) cDNA in *Heteropneustes fossilis* is done using random amplification method and the analysed result showed 1132 nucleotides that encoded 200 amino acids of ST (Anathy, Venugopal, Koteeswaran, Pandian & Mathavan, 2001). The result also showed that the *Heteropneustes fossilis* GH taxonomic relationship with other catfishes for 98% homology (Anathy et al., 2001). The successfulness of the GH expression in the vector and zebrafish proved that its clones viability of function.

2.4. Description of Somatotropin Gene

2.4.1. Size of Somatotropin Gene in *Tor tambroides*

The data in National Center for Biotechnology Information (NCBI) showed that *Tor tambroides* somatotropin (ST) gene is consisted of 633 nucleotides (Haini et al., 2011). The gene also encoded for 210 amino acids.

2.4.2. Structure of Somatotropin Gene in *Tor tambroides*

As mentioned in NCBI, somatotropin gene in *Tor tambroides* is constructed by:

a) Somatotropin-like region that is 181 in length (from 28 to 208 region) and related to placental lactogen and pituitary gland hormones in the basis of similar structure and function.
b) 34 receptor binding interface (polypeptide binding) that is 181 in total length and located at 28 to 208 region.

c) Signal peptide at N-terminus of the gene that is 22 in length and located at 1 to 22 region.

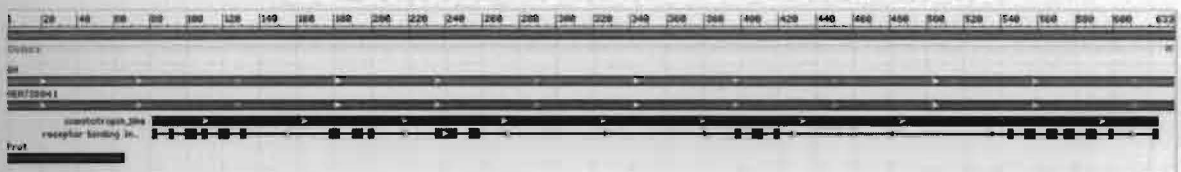


Figure 1. Graphical view of *Tor tambroides* somatotropin gene. (National Center for Biotechnology Information [NCBI], n.d. a).

2.4.3. Mechanism of Signal Transduction in Somatotropin Gene

The insulin-like growth factor 1 (IGF-1) is a complex that mediate the somatotropin (ST) (Clemmons, 2014). IGF-1 is usually bound to a highly affinity binding protein in the serum. The major regulations for ST are described in Table 1.

Table 1. Site of synthesize, medium of transportation and regulation of somatotropin. (Clemmons, 2014).

Site of synthesize	Medium of transportation	Regulation
Liver	Blood	Based on somatotropin level
Peripheral tissues	Surrounding cells	Autocrine or paracrine control and based on somatotropin level

As the synthesizing sites such as pituitary gland release the signals of ST to the liver, the liver will secrete IGF-1 to the blood system and mediate by the blood to targeted tissues such as the bone and muscle and then the action of ST will take place.

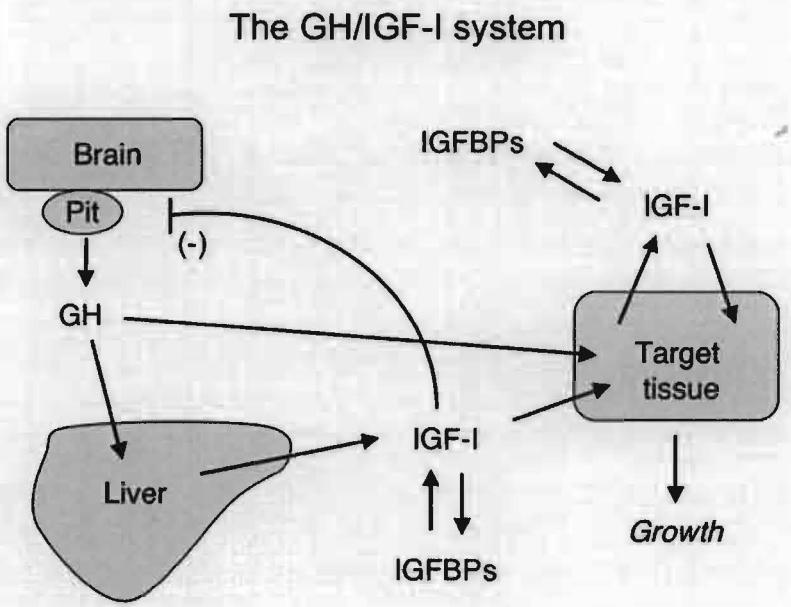


Figure 2. The somatotropin/insulin-like growth factor-1 system. (Shimizu, n.d.).

2.4.4. Decline in Somatotropin Consequences

Under normal condition, lacking in somatotropin (ST) will cause lessen in muscle mass as ST helps to increase the muscle mass (Stibich, 2014). In starvation condition, the growth rate also slower even though ST level is high because it is used to transport fatty acid and glycerol from the adipose storages (Sumpter et al., 1991).

2.4.5. Somatostatin

Somatostatin will affect the growth rate of organism by manipulating the insulin-like growth factor 1 (IGF-1) system that inhibit the pituitary somatotropin (ST) from synthesizing and secreting ST, and reduce ST and IGF-1 sensitivity (Sheridan & Hagemeister, 2010). Somatostatin is synthesized in neuroendocrine, during inflammatory action and immunology response (Patel, 1999).

2.5. Comparisons of *Tor tambroides* Somatotropin Gene with Other Species

The comparisons of *Tor tambroides* somatotropin (ST) gene with other teleosts species in NCBI database through BLAST system showed the percentage of similarity in ST gene conserved region. The similarity is listed in Table 2.

Table 2. Comparisons of *Tor tambroides* somatotropin gene with other species. (NCBI, n.d. a)

Species description	Query cover	Percentage of similarity
Hypsibarbus wetmorei ST	100%	99%
Cyprinus carpio ST	100%	98%
Cyprinus carpio ST 1	100%	98%
Onychostoma macrolepis ST	98%	97%
Triploid *Carassius cuvieri x* allotetraploid *hybrid* ST	100%	97%
Carassius auratus x Cyprinus carpio x Carassius cuvieri ST	100%	97%
Carassius auratus ST II	100%	97%

3. Materials and Methods

3.1. Total RNA Isolation

Muscle tissue of *Tor tambroides* was washed with 1X phosphate buffered saline. Then, the tissue was added with 1 mL of TRI Reagent® (Ambion, USA), homogenized and incubated at room temperature for 5 minutes. Homogenized sample was centrifuged at 12000 x g for 10 minutes at 4°C and the supernatant was transferred to a fresh tube. 200 µL of chloroform was added, well mixed and incubated at room temperature for 5 minutes. The sample was centrifuged at 12000 x g for 15 minutes at 4 °C and the upper layer (aqueous phase) was pipetted into a fresh tube. 500 µL of isopropanol was added, mixed and incubated at room temperature for 10 minutes. The tube was centrifuged at 12000 x g for 10 minutes at 4 °C and the supernatant was discarded. 1 mL 75% ethanol was added and inverted gently. After that, the tube was centrifuged at 7500 x g for 5 minutes at 4 °C. The ethanol was removed using pipette and air dried. The dried RNA pellet was resuspended in 30 µL ultrapure water. The isolated RNA was run on 1% (w/v) AGE at 60 V for 45 minutes.

3.2. Spectrophotometric Analysis of RNA

The RNA was transferred into microcuvette and the absorbance at 260 nm and 280 nm was taken. The absorbance ratio of A_{260}/A_{280} was calculated and the RNA purity was estimated based on Table 3.

Table 3. The range of estimation for RNA purity based on absorbance ratio of A_{260}/A_{280}. (Chomczynski & Sacchi, 2006).

Analysis	Ratio of A_{260}/A_{280}
Pure RNA preparation	1.8 to 2.0
RNA preparation containing impurities	< 1.8

3.3. Reverse Transcription Polymerase Chain Reaction

Components as shown in Table 4 were mixed and incubated at 65 °C for 5 minutes.

Table 4. RNA/primer mixture (1 reaction)

Components	Volume
Total RNA of 0.5 µg/µl concentration	*n* µL
Primer (50 ng/µL random hexamers)	1 µL
dNTP mix (10 mM)	1 µL
DEPC-treated water	Up to total volume of 10 µL

Then, the mixture was placed into ice cold temperature for 1 minute. 10 reactions of cDNA Synthesis Mix were prepared as shown in Table 5.

Table 5. cDNA Synthesis Mix (10 reactions)

Components	Volume
10X RT buffer	20 µL
$MgCl_2$ (25 mM)	40 µL
DTT (0.1 M)	20 µL
RNaseOUT™ (40 U/µL)	10 µL
SuperScript® III RT (200 U/µL)	10 µL

Negative control was prepared except that SuperScript® III RT (Invitrogen, USA) was not added but being replaced with ultrapure water. 10 µL of the cDNA Synthesis Mix was added to each RNA/primer mixture, gently mixed and briefly centrifuged. The mixture was incubated at 25 °C for 10 minutes, 50 °C for 50 minutes and terminated at 85 °C for 5

minutes. After that, the mixture was chilled on ice and briefly centrifuged. 1 µL of RNase H was added to each tubes and incubated at 37 °C for 20 minutes. The cDNA was stored at -20 °C.

3.4. Primer Designation

The nucleotides sequence of *Tor tambroides* somatotropin gene was obtained from GenBank of National Center of Biotechnology Information (NCBI). The sequence was analysed using Primer3Plus to find the forward and reverse primers. 1 forward primer and 5 different reverse primers were designed and checked for the parameters optimization (refer to Appendix A (b)).

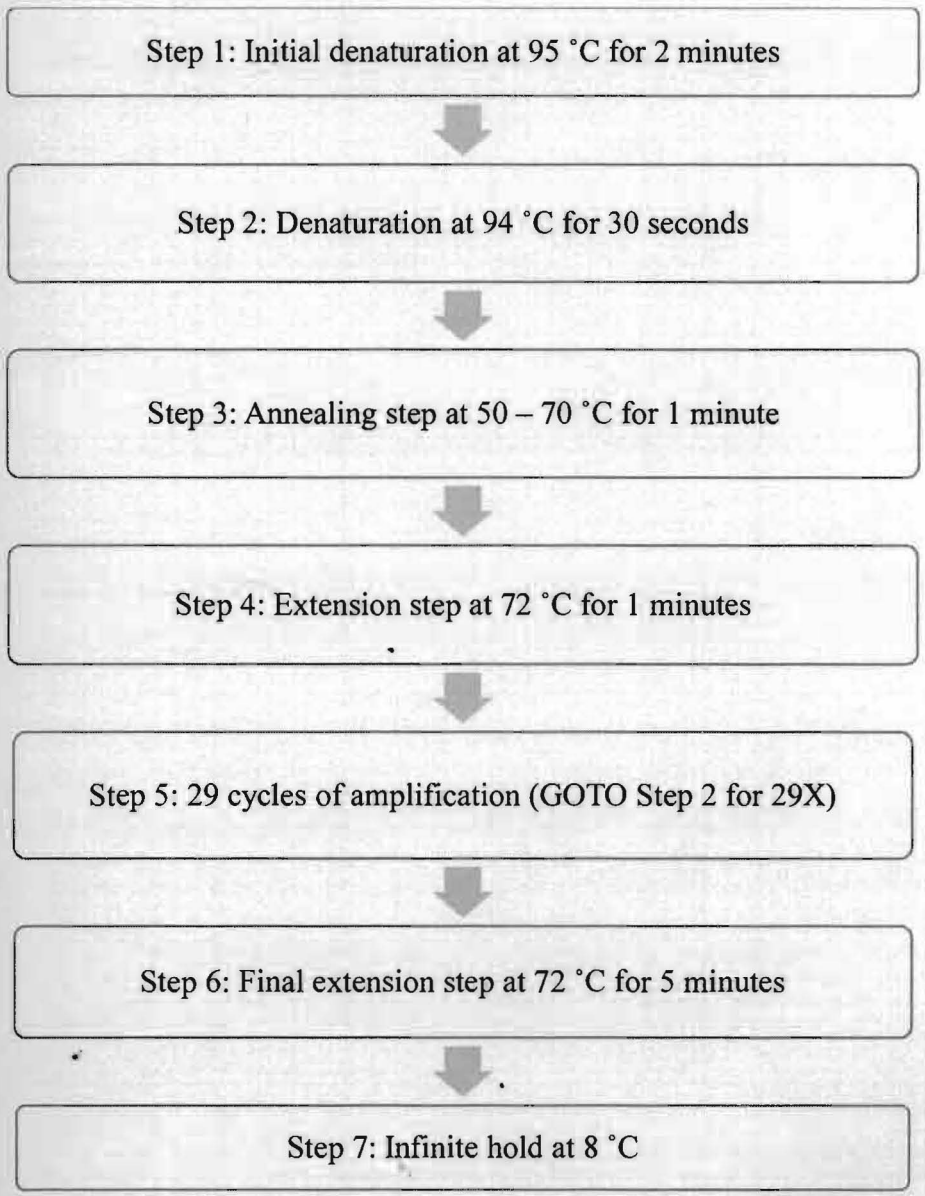
3.5. Polymerase Chain Reaction Optimization

A total of 5 PCR Master Mix that consist of 5 different reverse primers each were prepared as shown in Table 6. Each of the primer pairs PCR Master Mix were prepared in 8 reactions.

Table 6. PCR Master Mix

Components	1X	8X
Ultrapure water	14.8 µl	118.4 µl
5X reaction buffer	5.0 µl	40.0 µl
dNTP mix (10 µl of each dATP, dTTP, dGTP, dCTP)	0.5 µl	4.0 µl
25 mM of $MgCl_2$	1.5 µl	12.0 µl
Forward Primer (10 µM)	1.0 µl	8.0 µl
Reverse Primer (10 µM; Reverse 1, Reverse 2, Reverse 3, Reverse 4, Reverse 5 respectively)	1.0 µl	8.0 µl
cDNA	1.0 µl	8.0 µl
Taq DNA polymerase	0.2 µl	1.6 µl

A total volume of 25 µl per reaction was aliquoted to a fresh tube and labelled according to the type of primer and temperature. Negative control was prepared except that cDNA was not added but being replaced with ultrapure water. The gradient PCR was conducted under the following condition:



The PCR products were run on 1% (w/v) AGE at 60 V for 45 minutes and viewed under UV transilluminator. Then, the product was undergo PCR step again to get a clearer band.